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KI-67 PROLIFERATION INDEX IN NEUROENDOCRINE TUMORS OF GI SYSTEM ; A COMPARISON OF TWO COUNTING METHODOLOGIES.

Pathology

Smitirupa Mishra Resident, Deparment of pathology, Santokba durlabhji memorial hospital, Jaipur, Rajasthan.

Mitunkumar Mayani Senior Resident, Deparment of pathology, Santokba durlabhji memorial hospital, Jaipur, Rajasthan.

Shubha Gupta Honorary Consultant, Deparment of pathology, Santokba durlabhji memorial hospital, Jaipur, Rajasthan.

ABSTRACT

Background: The Ki67 index is important for grading neuroendocrine neoplasms. However, different counting methods exists:(1) 'eye-ball' estimation (2) automated counting by image analyzer (3) manual eye-counting (eye under microscope without a grid (4) manual count of digital camera captured/ colour printed image. The objective of the current study was to compare traditionally followed; Manual counting of Ki-67 positive cells under microscope and recently advocated; Manual counting of Ki-67 positive cells in digital camera captured and colour printed image. **Methods:** Ki67 immunostaining was performed on 62 resected neuroendocrine tumnors of GI system. Two methods of quantification were employed : i. Manual counting of Ki-67 positive cells in 500 tumor cells by the reporting histopathologist under microscope, usingx20 magnification in tumor 'hotspots'. Only strong nuclear staining was considered to be positive for Ki-67. Light brown or pale staining were ignored (M1). ii. Manual counting of Ki-67 positive cells in 500 tumor cells by the reporting histopathologist using x20 magnification in digital camera captured and colour printed image in tumor'hotspots'(M2). Comparison was based on multiple parameters viz cost effectiveness, reproducibility, practical ease of counting, time required for counting, limitations of individual methods and areas of discordance without compromising in therapeutic and prognostic efficacy. **Results:** When M1 and M2 grades were compared, 95.16% cases showed agreement and 4.84%cases showed Disagreement. Discordance in Ki67 index was seen near the cut-off values of G1-G2 tumors. Mean time taken for Ki-67 indexing by M1 (36.49 sec) was significantly less compared to mean time taken by M2 (7.11 min). The τ value obtained by M1 and M2 was 0.811; P <0.0001 and 0.628; P<0.0001 respectively. Near perfect interobserver reliability was obtained in both the methods with ICC of 0.9811 by M1 and ICC of 0.9860 by M2. The κ values for M1 shows moderate level of interobserver agreement (κ = 0.416, 95% CI=0.272-0.559) and κ values for M2 shows substantial agreement(κ = 0.697, 95% CI=0.565-0.828). The CCC for both the methodologies showed nearperfect correlation between the two histopathologists. M2 with CCC of 0.9719 (95%CI=0.9540 to 0.9829) had slightly better correlation than M1 with CCC of 0.9624 (95% CI=0.9409 to 0.9761).The concordance of Ki-67 proliferation index between M1 and M2 was excellent in all cases analysed with ICC of 0.9617 (95% CI: 0.9366 to 0.9769) and a CCC of 0.9252 (95% CI: 0.8789 to 0.9542). The cohen κ value for interobserver agreement between M1 and M2 was 0.891 (95% CI: 0.770 to 1.000) which shows near perfect agreement in grading by both the methodologies. **Conclusion:** It is important to assess Ki-67 indexing in the "hot-spot" area with the highest proliferative activity with a minimum count of 500 tumor cells. Majority of cases showed agreement in grading by both the methodologies. Disagreement was seen in G1-G2 tumors with Ki-67 values near the grade cut-offs. Ki-67 indexing by manual counting of camera captured/color printed image may require additional cost and time but is highly reproducible, reliable and accurate. Ki-67 indexing by manual count under a microscope seems very practical in theory but is not easy to employ as cell distribution and overlapping of cells make the test difficult to perform resulting in interobserver variability.

KEYWORDS

INTRODUCTION

Neuroendocrine neoplasms (NENs) arise from the diffuse neuroendocrine cell system and may occur at many different disease sites. Most frequently, these neoplasms occur in digestive system, followed by the lung.[1]Gastroenteropancreatic NETs (GEP-NETs) include carcinoid tumors of gastrointestinal tract and pancreatic NETs (pNETs). Whereas carcinoid tumors originate from enterochromaffin cells of the gut, pNETs are thought to arise in the islets of Langerhans.[2]

A three tiered grading system for GEP NEN based on Ki67 index (G1: ≤2%, G2: 3-20%, G3: >20%) or mitotic count has been introduced by the European Neuroendocrine Tumor Society (ENETS) and recently implemented in the WHO classification.[3] NETs are divided into three groups (NET G1,NET G2 and NET G3), whilethe specification G3 has been removed fromNECs (which are by definition high grade neoplasms), to avoid confusion with NET G3. This approach has proven to be of great help for prognostic stratification of patients and it is particularly useful for the distinction among the G3 neoplasms (Ki67>20%) and between NET G3 and NEC.[4]

There are various methodologies for counting Ki67 index- (1) 'eye-ball' estimation; it is carried out by scanning or 'eye-balling' the entire slide at x10 objective without actually counting individual cells.(2) automated counting by image analyzer; Ki-67 labeling index is obtained using an automated cellular image cytometer (ACIS® III) by Dako corporation (3) manual eye-counting (eye under microscope without a grid); This method consists of 'real-time' counting of Ki67-positive cells/500 or more tumor cells within a microscopic field in the identified 'hot spot' in x20 power (4) manual count of digital camera captured/ colour printed image; The area of greatest Ki67 positivity (hot spot) is selected for photographing and colour printing on white paper. Ki67 positive and negative tumor cells were then visualized and immediately circled/crossed.[5]

Having a well-established immunohistochemistry laboratory in the department, the present study was designed to grade the GEP-NENs according to WHO 2019 classification by Ki-67 indexing and to compare two methodologies (traditionally followed: manual counting under microscope v/s recently advocated: manual counting on camera captured image) on multiple parameters viz cost effectiveness, reproducibility, practical ease of counting, time required for counting, limitations of individual methods and areas of discordance without compromising in therapeutic and prognostic efficacy.

MATERIALAND METHODS

Comparative analysis type of study was conducted over a period of 18 months, extending from November 2020 to April 2022, in the department of Pathology in a tertiary care hospital in Northwest India .The study group comprised of All surgically resected specimen of GI System, diagnosed as NEN by IHC, irrespective of age and gender, received in the department consecutively during the study period. The study was approved by institutional ethics committee and subjects were included in study after written informed consent was obtained from the participants.

The following exclusion criteria were applied:

1. Cases histomorphologically suspicious of NEN but IHC study not requested.
2. Outside blocks of cases diagnosed as NEN or suspicious of NEN received for review.

IHC- After fixing specimen in buffered 10% formalin for 12 to 24

hours, grossing was done and details were recorded.Processing, paraffin embedding, section cutting (3-4 µm) and staining were done by standardized methods in the department. Slides were stained by Hematoxylin (Meyer's) and Eosin.[6] H&E stained slides were examined for histomorphology of tumor.Theblock best representative of the tumor was selected for immunohistochemical staining. Antigen retrieval was done by Heat Induced Epitope Retrieval method by BIO GENEX-EZ Retriever system V.3 and 100x Tris-EDTA buffer at pH 9.0 (100mM Tris + 10mM EDTA) was used for the above.

Methods of Ki-67 Quantification[5]-

i. Manual counting of Ki-67 positive cells in 500 tumor cells by the reporting histopathologist under microscope,usingx20 magnification in tumor 'hotspots'. Only strong nuclear staining was considered to be positive for Ki-67. Light brown or pale staining were ignored (M1).
ii. Manual counting of Ki-67 positive cells in 500 tumor cells by the reporting histopathologist using x20 magnification in digital camera captured and colour printed image in tumor'hotspots'. Slides were scanned using Olympus BX41 light microscope and images were captured using Magnus Magcam MU2A digital camera with 2.3 megapixel CMOS chip (Figure 6). Images were printed in color at a size of 8 inches in height by 10 inches in width, at 96 dots per inch resolution. Ki-67 positive and negative tumor cells were circled / crossed off for counting.Only strong nuclear staining was considered to be positive for Ki-67. Light brown or pale staining were ignored(M2).
iii. Both the counting methodologies were performed by two histopathologists (P1 and P2) and time taken for Ki-67 indexing was recorded for individual histopathologist.

All tumors were graded according to WHO 2019 classification of NEN. In each case, Ki-67 index counting was done by two consultant histopathologists. Only strong nuclear staining was considered to be positive for Ki-67. Light brown or pale staining were ignored.

Stastical Analysis

Medcalc 16.4 version software was used for all statistical analysis. The Kendall tau-b correlation coefficient was used to determine the relationship between the amount of time spent in assessing the Ki-67index and the quantification of Ki-67 index by both the methodologies. "0" indicates no correlation and "1" indicates perfect positive correlation.

Intraclass correlation coefficient (ICC) was used to assess the interobserver reliability of Ki-67 index by both the methods. Here the ICC reflects a scale of reliability measurement of two observers, where 1.0 corresponds with perfect reliability and 0.0 represents no reliability. The Cohen κ was used to evaluate Ki-67 count agreement between within groups. The κ values may be interpreted as 0-0.2 representing slight agreement, 0.2-0.4 fair, 0.4-0.6 moderate, 0.6-0.8 substantial and >0.8 almost perfect agreement. The concordance of Ki-67 index(CCC) obtained by both the pathologists by M1 and M2 was assessed. Perfect correlation was indicated by 1 and absence of any correlation was indicated by 0.

RESULTS

62 Cases of NEN of GI system satisfied the inclusion criteria and were included in the study for evaluation.

When the cases were graded according to Method 1(M1) :43(69.35%) cases were of grade 1 (Ki-67 index =<3%), 16(25.81%) cases were of grade 2 (Ki-67 index = 3-20%) and 3(4.84%) cases were of grade 3 (Ki-67 index = >20%). Upon grading the cases by Method 2(M2); 44 (70.97%) cases were of grade 1, 15 (24.19%) cases were of grade 2 and 3 (4.84%) cases were of grade 3.

In grade 3 tumors, agreement was seen in 3 (100%) cases while comparing Method 1 and Method 2.In grade 1 and grade 2 tumors agreement was seen in 42 (97.67%) cases and 14 (87.50%) cases respectively while comparing both the methodologies. In 3/62 cases disagreement was seen; 1 case was upgraded from grade 1 to grade 2 by method 2 and 2 cases were downgraded from grade 2 to grade 1 by method 2.

Mean time taken by both the pathologist in M1 = 36.49 sec±14.27 SD; median = 33 sec; range=16.5-75 sec and Mean time taken by both the pathologist in M2 =7.11 min±4.32 SD; median = 6 min; range=2.51-22.77 min.

Kendall's tau-b

There was strong, positive correlation noted between time spent in scoring and the Ki-67 score for method 1 (kendall's tau-b=0.811;P<0.0001) and a positive correlation between time spent in scoring and the Ki-67 score for method 2 (kendall's tau-b=0.628;P<0.0001).

The level of agreement for Ki-67 index between pair of pathologist in method 1 was moderate (κ=0.416, 95% CI= 0.272-0.559) and in method 2 was substantial (κ=0.697, 95% CI=0.565-0.828).

Intra/Inter Class Correlation Coefficient (ICC)

- The ICC for Ki-67 index count between pathologist 1 and 2 by method 1was 0.9811(95% CI, 0.9687-0.9886) and by method 2 was 0.9860(95% CI, 0.9736-0.9914).
- ICC for grading by method 1 and method 2 was 0.9617; 95% CI=0.9366-0.9769.
- There was almost perfect agreement in grading by both the methodologies (κ=0.891, 95% CI=0.770-1.000)

Concordance Correlation Coefficient(CCC)

- The CCC for method 1 was 0.9624(95% CI=0.9409 to 0.9761) and for method 2 was 0.9719(95% CI=0.9540 to 0.9829).
- There was perfect correlation between grades of tumors derived by both the methodologies of Ki-67 indexing (CCC=0.9252; 95% CI=0.8789-0.9542).

DISCUSSION

The Ki-67 index is an established prognostic factor in gastrointestinal NENs and defines tumor grades.[7] However, its adaptation into daily practice has been fraught with challenges related to counting methodologies.

We, through this comparative study and analysis between two methods: manualcounting with microscope (M1) and manual counting of camera captured/printed image (M2), have attempted to come up with a suitable method of Ki-67 indexing considering the balance of reproducibility, practicality and accuracy.

The present study includes 62 cases of GEP-NENs for quantitative comparison of Ki-67 index as in studies done by Riedet al[5] and Satturwar et al[8] who considered 68 and 50 cases respectively for the same.

In our study we have compared two methodologies of Ki-67 indexing for grading of Neuroendocrine neoplasms (NEN) of GI System (according to WHO 2019 classification of NENs). Grading the tumors by M1 gave almost equal results as grading the tumors by M2. Majority of cases were of grade 1 (M1-69.35%; M2-70.97%) followed by grade 2 (M1-25.81%; M2-24.19%) and grade 3 (M1-4.84%; M2-4.84%) by both the methodologies. Similar results were obtained in studies done by Miller H C et al[3], Cottenden J et al[9],Jin M et al[10] andBasile M L et al.[11]

In study done by Shi H et al[12] which included 514 patients of GEP-NENs, 216 (42%) cases were of grade 3. As it was a retrospective study, willingly more grade 3 cases were selected as they wanted to observe variability of Ki-67 index in primary and metastatic site of GEP-NENs.

GEP-NENs: Analysis Of Agreement And Disagreement Between M1 & M2

Table 1: Percentage Of Agreement And Disagreement Results (M1 v/s M2)(N=62)

Description	In no.	In %
No. of cases showing agreement	59	95.16%
No. of cases showing disagreement	03	4.84%
Total number of cases	62	100%

- **Percentage Of Agreement (M1 v/s M2)**

After grading the cases by both the methodologies, % of agreement in between them was calculated. In our study 95.16% cases showed agreement amongst M1 and M2. This was in concordance with studies done by Satturwaret al[8], Dogukan F M et al[13]and Kroneman T L et al[14] who similarly found 96%, 95.87% and 97% agreement respectively among similar counting methodologies.

Studies done by Cottenden J et al[9], Dere Y et al[15] and Basile M L et al[11] show poor agreement: 55%, 70% and 65% respectively when compared with our study.

- **Grade Wise Analysis Of Percentage Of Agreement (M1 v/s M2)**

When tumor grade assessment was done by both the methods, agreement among G1 tumors was 97.67% and among G2 tumors was 87.50%. Similar observations were made in studies done by Basile M L et al[11] and Hwang H S et al[16] in which agreement among G1 tumors were comparatively more than G2 tumors. In study done by Dere Y et al[15],40% agreement was seen among G2 tumors which was significantly more than 14% agreement among G1 tumors.

- **Percentage Of Disagreement (M1 v/s M2)**

In our study 4.84% cases showed disagreement amongst M1 and M2. This was in concordance with studies done by Satturwar et al[8], Dogukan F M et al[13] and Kroneman T L et al[14] who similarly found 4%, 4.13% and 3% agreement respectively among similar counting methodologies. Studies done by Cottenden J et al[9], Dere Y et al[15] and Basile M L et al[11] show higher levels of disagreement: 45%, 30% and 35% respectively when compared with our study.

Upgrading/Downgrading Of Cases Showing Disagreement(Table 2)

Table 2: Analysis Of Discordant Cases In WHO Grades Between M1& M2 (N=3)

Case No.	Accession no.	Ki-67 index (M1)			Grade (M1)	Ki-67 index (M2)			Grade (M2)
		P1	P2	Mean		P1	P2	Mean	
22	17897	3%	3%	3%	G2	2%	2%	2%	G1
44	94969	3%	3%	3%	G2	2%	2%	2%	G1
46	97107	2%	2%	2%	G1	3%	3%	3%	G2

In our study, disagreement was seen in 3/62 cases, of which 2 cases were downgraded by M2 and one case was upgraded by M2. In studies done by Satturwar et al[8], Kroneman et al[14] and Basile M L et al[11] most of the cases were downgraded and moreover the manual count on camera captured/printed image method had a tendency to downgrade the tumors which is also noted in our case. In study done by Dere et al[15], among the reclassified cases most of them were upgraded which may be due to the use of a self-designed software of their institute for Ki-67 counting.

Area Of Disagreement Among The Reclassified Cases (Table 2)

In our study discordance was seen among G1-G2 tumors which had Ki-67 values near to the grade cut-offs. Similar observations were made in study done by Satturwaret al[8], Kroneman T N et al[14] and Tang L H et al[17] in which disagreement were noted only among grade 1 and grade 2 tumors.

The τvalue obtained by M1 and M2 was 0.811; P <0.0001 and 0.628; P<0.0001 respectively. This suggested a strong and positive correlation between time spent scoring and the Ki-67 score for both the methodologies. Satturwar et al[8] in their study calculatedτvalues for manual Ki-67 assessment methods which also had a positive correlation(τ=0.221; P=0.024).

In our study ICC was used to assess the interobserver reliability of Ki-67 index by both the methods. Near perfect reliability was obtained in both the methods with ICC of 0.9811 by M1 and ICC of 0.9860 by M2. Interobserver reliability was slightly more in M2. Similar results were obtained in study done by Cottenden J et al[9] and Polley et al[18].

The κ values for M1 shows moderate level of interobserver agreement (κ = 0.416, 95% CI=0.272-0.559) and κ values for M2 shows substantial agreement (κ=0.697,95% CI=0.565-0.828).These results were in concordance with study done by Jin M et al[10] and Voros A et al[19].

The CCC for both the methodologies showed near perfect correlation between the two histopathologists. M2 with CCC of 0.9719 (95%CI=0.9540 to 0.9829) had slightly better correlation than M1 with CCC of 0.9624 (95% CI=0.9409 to 0.9761). Similar results were observed in study done by Kroneman T N et al[14] and Tang L H et al.[17]

The concordance of Ki-67 proliferation index between M1 and M2 was excellent in all cases analysed with ICC of 0.9617 (95% CI: 0.9366 to 0.9769) and a CCC of 0.9252 (95% CI: 0.8789 to 0.9542). The results were similar to studies done by Desmeules P et al[20], Saadeh H et al[21] and Polley et al[18].

The cohen κ value for interobserver agreement between M1 and M2 was 0.891(95% CI: 0.770 to 1.000) which shows near perfect agreement in grading by both the methodologies. Our study was in concordance with study done by Voros A et al[19] which showed substantial agreement between the counting methodologies.

CONCLUSION

On grading the GEP-NENs and comparing the Ki-67 index counting methodologies: manual counting under microscope v/s manual count on camera captured/printed image, the present study concludes:

It is important to assess Ki-67 indexing in the "hot-spot" area with the highest proliferative activity with a minimum count of 500 tumor cells. Ki-67 indexing by manual counting of camera captured/color printed image may require additional cost and time but is highly reproducible, reliable and accurate. Ki-67 indexing by manual count under a microscope seems very practical in theory but is not easy to employ as cell distribution and overlapping of cells make the test difficult to perform resulting in interobserver variability. Majority of cases (95.16%) showed agreement in grading by both the methodologies.

Disagreement was seen among 4.84% cases in G1-G2 tumors with Ki-67 values near the grade cut-offs. Manual counting on camera captured images had a tendency to downgrade the cases.

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